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Cytospora from *Salix* in northern China

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Abstract — Fungal specimens from *Salix* causing willow *Cytospora* canker in northern China were evaluated through morphological observation and phylogenetic analysis. They were identified as four species — *C. rostrata* sp. nov., *C. nivea*, *C. chrysosperma*, and *Valsa populina*. *Cytospora rostrata* is a new beaked species parasitizing *Salix cupularis* in Gansu Province, China. It is morphologically distinguished from other *Cytospora* species by its fruiting bodies with a single locule and a thorn-like beak. Molecular evidence obtained from the internal transcribed spacer (ITS) region also suggests that *C. rostrata* represents a distinct lineage within *Cytospora*. This is the first Chinese record of *C. nivea* on *Salix*.

Key words — *Diaporthales*, morphology, phylogeny, taxonomy

Introduction

Willow (*Salix*) is a crucial commercial and ecologically important forest tree that is grown as a major biomass energy source for its yield potential and coppicing ability in Netherland (Londo 2002). In China, willows are one of the most important trees for their economic and ecological value, and are also widely planted in courtyards, residential districts, and parks as a highly adversity-resistant perennial tree. However, most willow trees are under serious threat in northern China from dieback and stem canker caused by *Cytospora* species. A systematic study of pathogenic fungi on *Salix* is badly needed.

The genus *Cytospora* was established by Ehrenberg in 1818 and has been shown to be the anamorph of the ascomycetous genus *Valsa* Fr. (*Diaporthales*) (Adams et al. 2005), causing serious dieback and stem canker diseases commonly found on woody plants (Schreiner 1931, Spielman 1984, Wang et al. 2014). About 110 species of *Cytospora* have been described on more than 85 host species (Adams et al. 2005, Kirk et al. 2008). Traditional taxonomy

of *Cytospora* has been based heavily on host species, but Adams et al. (2006) and Mehrabi et al. (2011) have recently stressed the taxonomic significance of morphology (including presence or absence of a conceptacle, number and arrangement of locules, size and shape of conidiophores, and conidial size) in this group.

Twenty-six species of *Cytospora* and *Valsa* have been recorded as pathogenic fungi of willow canker worldwide, of which nine have been reported in China: *C. capreae* Fuckel, *C. salicis* (Corda) Rabenh., *C. fugax* (Bull.) Fr., *C. fertilis* Sacc., *C. translucens* Sacc., *C. germanica* Sacc., *C. chrysosperma*, *C. ambiens* (Pers.) Sacc., and *V. populina* (Deng 1963, Tai 1979, Wei 1979, Zhuang 2005). However, morphological data for some of these records were inadequate for identification, and taxonomic studies of *Cytospora* species from *Salix* in China are lacking. We therefore collected 116 isolates from ca. 200 specimens on willow trees from nine provinces of northern China and used 14 representative isolates in a systematic study based on morphology and DNA sequence variation. Our specimens included a new taxon, which we describe as *Cytospora rostrata*.

Materials & methods

Sample collection and isolation

A total of 116 *Cytospora* isolates were obtained from lesions on infected twigs of willow trees in nine Chinese provinces: Xinjiang, Tibet, Shaanxi, Gansu, Ningxia, Qinghai, Heilongjiang, Beijing, and Liaoning. Plant tissues were cut into ca. 5 mm diameter discs, placed in 70% ethanol for 1 min, and then rinsed with sterile water three times. The twig segments were then placed onto the surface of potato dextrose agar (PDA) and incubated at 28°C. The growing edges of hyphae were transferred onto a new PDA plate after 1–2 days. Each isolate was purified through single spore isolation and maintained according to Fan et al. (2014).

Morphological observation

Cytospora specimens used for morphological observation have been conserved in the Museum of Beijing Forestry University (BJFC). Morphological characteristics of the fruiting body, including the size of stromata, color, size and shape of disc, presence or absence of a conceptacle, number and diameter of ostioles per disc, arrangement of locules, and conidiophore size and shape, were recorded. Over 20 conidiomata were sectioned and diameters of 50 conidia were randomly chosen to measure the length and width by a Leica light microscope (DM 750). Cultural characteristics including colony color and pycnidial structure were recorded after 3, 7, and 30 days.

DNA extraction and PCR amplification

Genomic DNA of all cultures was extracted using a modified CTAB method (Doyle & Doyle 1990). Extracted DNA was electrophoresed on 1% agarose gel to check genomic DNA quality. The ITS1-5.8S-ITS2 (ITS) region of the rDNA was amplified by PCR using the primer pair ITS1 and ITS4 (White et al. 1990) in 20 µL volumes with 1 µL (20 ng) DNA template, 1.6 µL dNTP (2.5 mmol/L), 2 µL 10×PCR buffer (15 mM/mL $MgCl_2$),

Table 1. New ITS sequences of *Cytospora* and *Valsa* spp. included in phylogenetic analyses.

Species	Host	Location	Isolate	Sequence
C. rostrata	*Salix cupularis*	China, Gansu	BJFC CGLs251 **HT**	KC313890
	S. cupularis	China, Gansu	BJFC CGLs252	KC313891
	S. cupularis	China, Gansu	BJFC CGLs280	KC313892
C. chrysosperma	*S. babylonica*	China, Ningxia	BJFC CNLs2	KC787357
	S. babylonica	China, Shaanxi	BJFC CSLs3	KC787358
	S. carmanica	China, Gansu	BJFC CGLs4	KC787359
	S. cupularis	China, Gansu	BJFC CGLs8	KC787360
	S. matsudana	China, Gansu	BJFC CGLs2	KC787361
	S. matsudana f. *tortuosa*	China, Gansu	BJFC CGLs7	KC787362
	S. babylonica	China, Ningxia	BJFC VNLs2	KC787365
C. nivea	*S. cupularis*	China, Gansu	BJFC CGLs32	KC787364
	S. cupularis	China, Gansu	BJFC VGLs33	KC787363
V. populina	*S. matsudana* f. *tortuosa*	China, Gansu	BJFC VGLs7	KC787366
	S. cupularis	China, Gansu	BJFC VGLs24	KC787367

HT = holotype.

1 μL of each primer (10 μLmol/L), 0.1 μL Taq DNA polymerase (5 U/μL), and 13.3 μL ddH_2O. The PCR protocol comprised denaturation at 94°C for 10 min, followed by 35 cycles of 94°C for 30 s, 51°C for 30 s, 72°C for 40 s, and a final cycle at 72°C for 10 min. PCR products were electrophoresed on 1% agarose gel and the DNA was sequenced by the Shanghai Invitrogen Biological Technology Corporation.

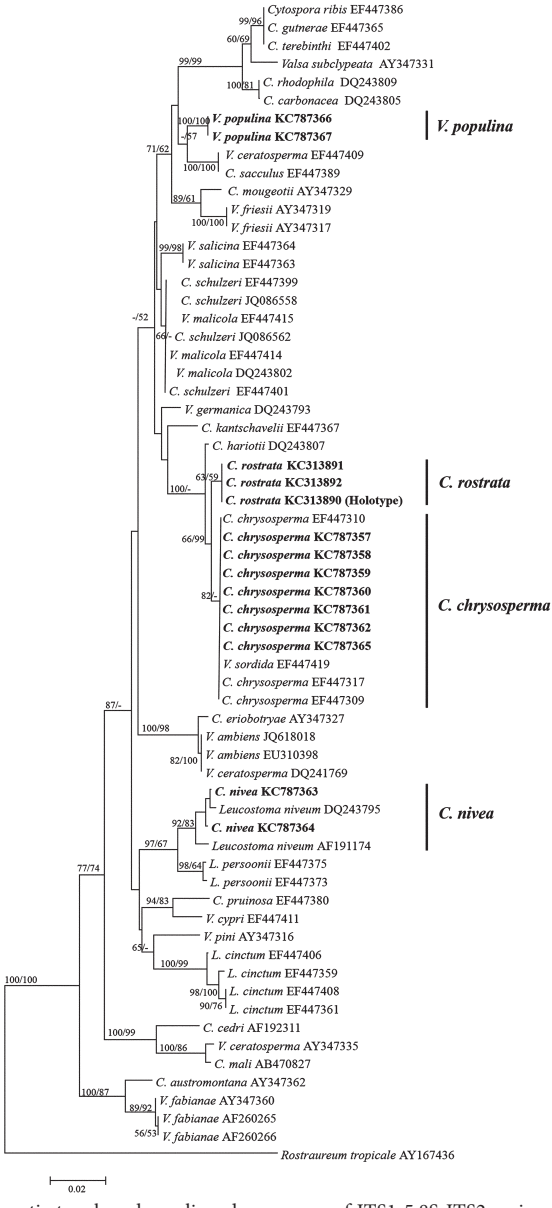
Phylogenetic analyses

Our 14 sequences representing the four species isolated from *Salix* were used in the phylogenetic analyses (Table 1) with 49 GenBank ITS sequences representative of a cross-section of *Cytospora*/*Leucostoma*/*Valsa* spp., and *Rostraureum tropicale* Gryzenh. & Wingf. as outgroup. The sequences were assembled using SeqMan (7.1.0) and aligned using Clustal X (1.81). Alignments were manually adjusted to allow maximum alignment and maximum sequence similarity, and gaps were treated as missing data. The phylogenetic tree was constructed with the neighbor-joining (NJ) method using PAUP (4.10), and the maximum likelihood (ML) method using Phylip (3.6.9) (Swofford 2000, Tamura et al. 2011). The stability of relationships was assessed by bootstrap analyses comprising 1000 data settings. Constructed trees were viewed by Treeview (1.6.6) software.

Results

Phylogenetic analyses

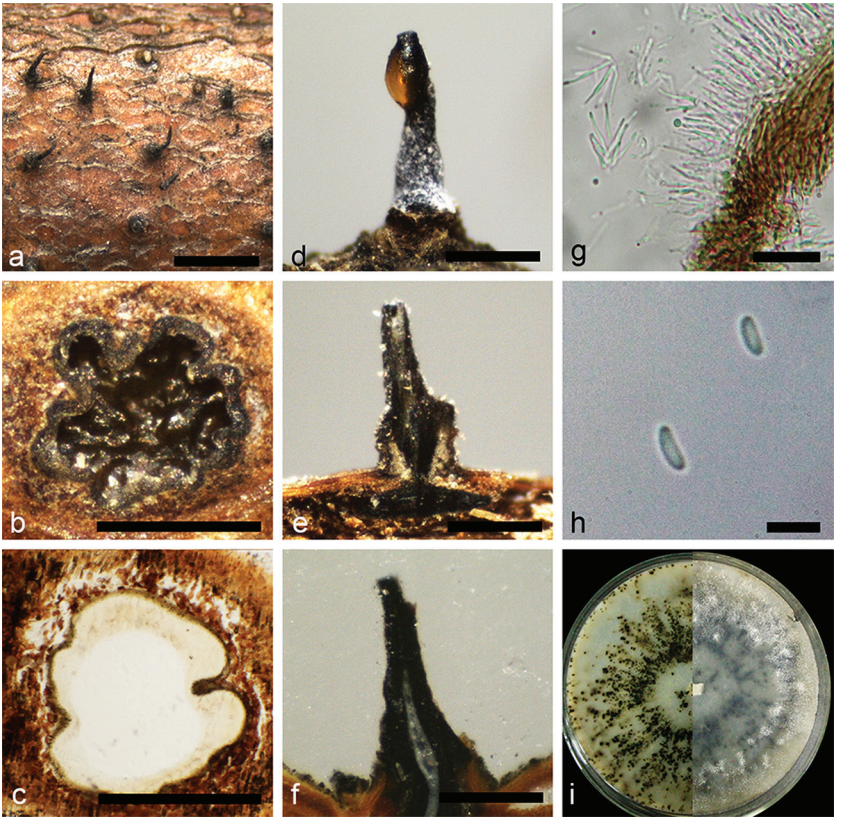
The alignment of ITS regions consisted of 510 base pairs. The ML analysis produced a similar topology to the NJ analysis presented in Plate 1.



Pl. 1 Phylogenetic tree based on aligned sequences of ITS1-5.8S-ITS2 region of 63 isolates of *Cytospora, Leucostoma,* and *Valsa* generated from neighbor joining (NJ) and maximum likelihood (ML) analyses. NJ and ML bootstrap values greater than 50% are indicated (separated by a slash from 1000 heuristic replicates). Tree is rooted with outgroup *Rostraureum tropicale*. The new sequences are represented in bold font.

The sequences grouped into 29 clades. *Cytospora rostrata* had a high similarity of several nucleotides variation with *C. chrysosperma*. However, the phylogenetic tree and the morphological characteristics indicated *C. rostrata* represents an independent taxon.

Taxonomy



Pl. 2 *Cytospora rostrata* (Holotype, BJFC-CGLs251). **a** Conidiomata on twig. **b, c** Transverse sections through conidiomata. **d** Detail of beak on twig. **e, f** Longitudinal sections through conidiomata. **g** Conidiophores and conidiogenous cells. **h** Conidia. **i** Colonies on PDA at 3 d (left) and 30 d (right). Scale bars: a = 1 mm; b–f = 0.5 mm; g = 20 µm; h = 5 µm.

Cytospora rostrata C.M. Tian & X.L. Fan, **sp. nov.** Plate 2

MycoBank MB 810728

Differs from *Cytospora chrysosperma* by its conidial stromata with a single simple locule and thorn-like ostiolar beak.

Type: **CHINA**, **Gansu Province**, Gannan, Diebu, 34°04′48.85″N 103°23′34.20″E, elev. 2989 m asl, on stems of *Salix cupularis* Rehder, 9 August 2012, Y.M. Liang & X.L. Fan (**Holotype**, BJFC-S726; culture BJFC-CGLs251; GenBank KC313890).

Etymology: *rostrata*, referring to the beaked fruiting body.

Conidiomatal stromata immersed in bark, erumpent, beaked, discoid to conical, with a single locule. Disc absent. Beak slim, spiny, 0.87–1.20 mm (x = 0.98, n = 20), with one ostiole per beak. Ostiole in the center of the beak, 22–24 μm (x = 23 μm, n = 20) in diameter. Locules discoid, simple-loculed, 455–851 μm (x = 668 μm, n = 20) in diameter. Conidiophores hyaline, unbranched or occasionally branched at the bases, 15.3–25.1 μm (x = 18.9 μm, n = 20). Conidia hyaline, eguttulate, elongate-allantoid, aseptate, 3.6–4.8 × 1.0–1.6 μm (x = 4.1 × 1.38 μm, n = 50). Cultural characteristics were white originally, became pale yellow after 6–7 d. The colony was flat, thin, and with a uniform texture. The arrangement of conidiomata on PDA was circular in the center and radial to colony edge.

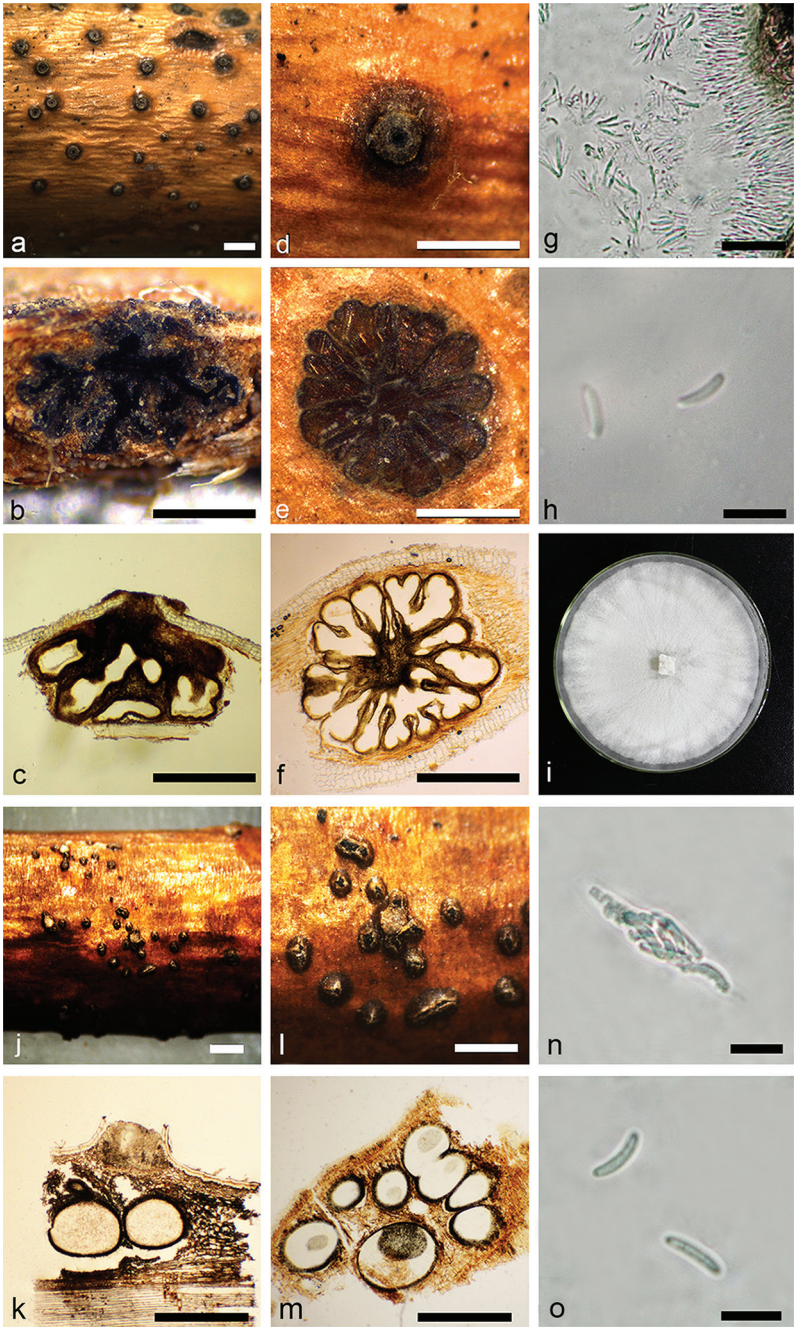
Teleomorph not found.

Additional specimens examined: **CHINA**, **Gansu Province**, Diebu, 34°04′48.35″N 103°23′36.60″E, elev. 2973 m asl, on stems of *Salix cupularis,* 9 August 2012, Y.M. Liang & X.L. Fan (BJFC-S727; culture BJFC-CGLs252; GenBank KC313891); Diebu, 34°04′44.65″N 103°23′39.42″E, elev. 2977 m asl, on stems of *Salix cupularis,* 9 August 2012, Y.M. Liang & X.L. Fan (BJFU-S733; culture BJFC-CGLs280, GenBank KC313892).

Cytospora chrysosperma (Pers.) Fr. Syst. Mycol. 2: 542, 1823. Plate 3

Conidiomata stromata immersed in bark, erumpent, discoid, flask-shaped to conical, with a large multiple locules. Disc grey to black, nearly flat, circular to ovoid, 0.21–0.33 mm (x = 0.22 mm, n = 20) in diameter, with one ostiole per disc. Ostiole medium grey, prominent, 62–85 μm (x = 76 μm, n = 20) in diameter. Locules complex multi-loculed, subdivided frequently by invaginations, sharing common walls, 0.64–1.26 mm (x = 1.01 mm, n = 20) in diameter. Conidiophores hyaline, unbranched or occasionally branched at the bases, 17.3–28.2 μm (x = 22.6 μm, n = 20). Conidia hyaline, eguttulate, elongate-allantoid, aseptate, 4.0–5.6 × 0.8–1.3 μm (x = 4.7× 1.2 μm, n = 50). Cultural characteristics were white originally, becoming partially pale yellow after 6–7 d although primarily white. The colony was flat, felt-like, and the texture was uniform. Conidiomata formed on PDA randomly.

Pl. 3 *Cytospora chrysosperma* (BJFC-CNLS2). **a** Conidiomata on twig. **b, c** Longitudinal sections through conidiomata. **d** Detail of beak on twig. **e, f** Transverse sections through conidiomata. **g** Conidiophores and conidiogenous cells. **h** Conidia. **i** Colonies on PDA at 3 d. **j, l** Habit of ascomata on twig. **k** Longitudinal sections through ascomata. **m** Transverse sections through ascomata. **n** Asci and immature ascospores. **o** Ascospores. Scale bars: a = 1 mm; b–f, j–m = 0.5 mm; g = 20 μm; h = 5 μm; n, o = 10 μm.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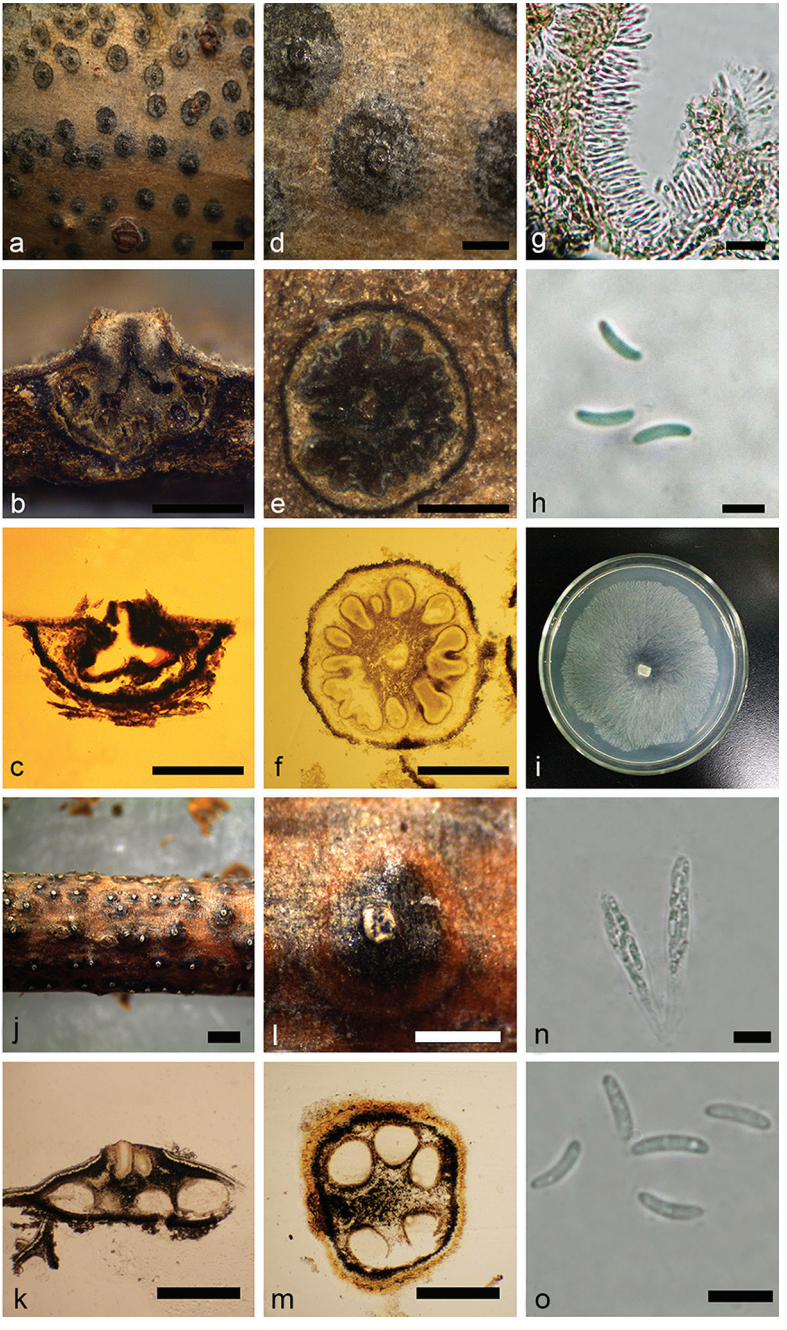
A huge ascostromatal region immersed in the bark, erumpent, extending basally up to 1.62–2.46 mm (x = 2.13 mm, n = 20) in diameter. Ascostromata usually scattered, erumpent, circular to ovoid, 0.86–1.19 mm (x = 0.97 mm, n=20) diameter. Disc was usually obscured by tightly ostiolar necks, when apparent pale brown to black, nearly hemispherical, circular to ovoid, 0.20–0.34 mm (x = 0.30 mm, n = 20) in diameter, with 4–8 ostioles arranged circinately in a disc, brown to black. 4–8 perithecia arranged circinately in black entostromata, flask-shaped to spherical, 0.26–0.34 mm (x = 0.30 mm, n = 20) in diameter. Asci free, clavate to elongate obovoid, 38.6–43.8 × 5.1–6.2 μm (x = 40.3 × 5.4 μm, n = 20), 8-spored. Ascospores, elongate-allantoid, thin-walled, hyaline, aseptate, 8.3–13.1 × 2.0–2.8 μm (x = 11.1 × 2.4 μm, n = 50). Cultural characteristics were white. The colony was flat, felt-like, and the texture was uniform. Conidiomata formed on PDA uniformly.

Materials examined: **China**, **Gansu Province**, Gannan, Lintan, Yeliguan, 34°57′36.48″N 103°39′55.12″E, elev. 2187 m asl, on stems of *Salix carmanica* Bornmüeller, 5 August 2012, X.L. Fan (BJFC-S702; culture BJFC-CGLs4; GenBank KC787359); Gannan, Zhuoni, Dayu National Forestry Park, 34°22′19.82″N 103°35′55.45″E, elev. 2181 m asl, on stems of *Salix cupularis*, 7 August 2012, X.L. Fan (BJFC-S706; culture BJFC-CGLs8; GenBank KC787360); Gannan, Lintan, Yeliguan, 34°57′43.41″N 103°39′44.82″E, elev. 2189 m asl, on stems of *Salix matsudana* Koidzumi, 5 August 2012, X.L. Fan (BJFC-S700; culture BJFC-CGLs2; GenBank KC787361); Gannan, Lintan, Yeliguan, 34°57′36.48″N 103°39′59.70″E, elev. 2187 m asl, on stems of *Salix matsudana* f. *tortuosa* (Vilm.) Rehder, 5 August 2012, X.L. Fan (BJFC-S705; culture BJFC-CGLs7; GenBank KC787362). **Ningxia Province**, Yinchuan, 38°31′51.80″N 106°17′48.05″E, elev. 1231 m asl, on stems of *Salix babylonica* L., 13 February 2012, X.L. Fan (BJFC-S508; culture BJFC-CNLs2; GenBank KC787357; culture BJFC-VNLs2; GenBank KC787365). **Shaanxi Province**, Weinan, Hua, Xiamiao, 34°34′47.66″N 109°45′41.61″E, elev. 335 m asl, on stems of *Salix babylonica* L., 26 May 2012, X.L. Fan (BJFC-S571; culture BJFC-CSLs3; GenBank KC787358).

Cytospora nivea (Hoffm.) Sacc., Michelia 2: 264, 1881, nom. illegit., non Fuckel 1860.
Plate 4

Conidiomata immersed in bark, erumpent, discoid, flask-shaped to conical, conceptacles dark, with a large multiple locules. Disc grey, nearly flat, became white when old, raised, circular to ovoid, 0.20–0.34 mm (x = 0.29 mm, n = 20) in diameter, with one ostiole per disc. Ostioles medium grey, prominent, 60–92 μm (x = 77 μm, n = 20) in diameter. Locules complex multi-loculed, subdivided frequently by invaginations, sharing common walls, 0.69–1.04 mm (x = 0.88

Pl. 4 *Cytospora nivea* (BJFC-CGLs32). **a** Conidiomata on twig. **b, c** Longitudinal sections through conidiomata. **d** Detail of beak on twig. **e, f** Transverse sections through conidiomata **g** Conidiophores and conidiogenous cells. **h** Conidia. **i** Colonies on PDA at 3 d. **j, l** Habit of ascomata on twig. **k** Longitudinal sections through ascomata. **m** Transverse sections through ascomata. **n** Asci and immature ascospores. **o** Ascospores. Scale bars: a, j = 1 mm; b–f, k–m = 0.5 mm; g = 20 μm; h = 5 μm; n, o = 10 μm.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mm, n = 20) in diameter. Conidiophores hyaline, unbranched or occasionally branched at the bases, 19.3–36.6 μm (x = 27.2 μm, n = 20). Conidia hyaline, eguttulate, elongate-allantoid, aseptate, 6.3–8.0 × 1.1–1.4 μm (x = 7.1 × 1.2 μm, n = 50). Cultural characteristics were white originally, became dark brown and black after 14 d. The colony was flat, felt-like, and the texture was uniform. Conidiomata formed on PDA uniformly.

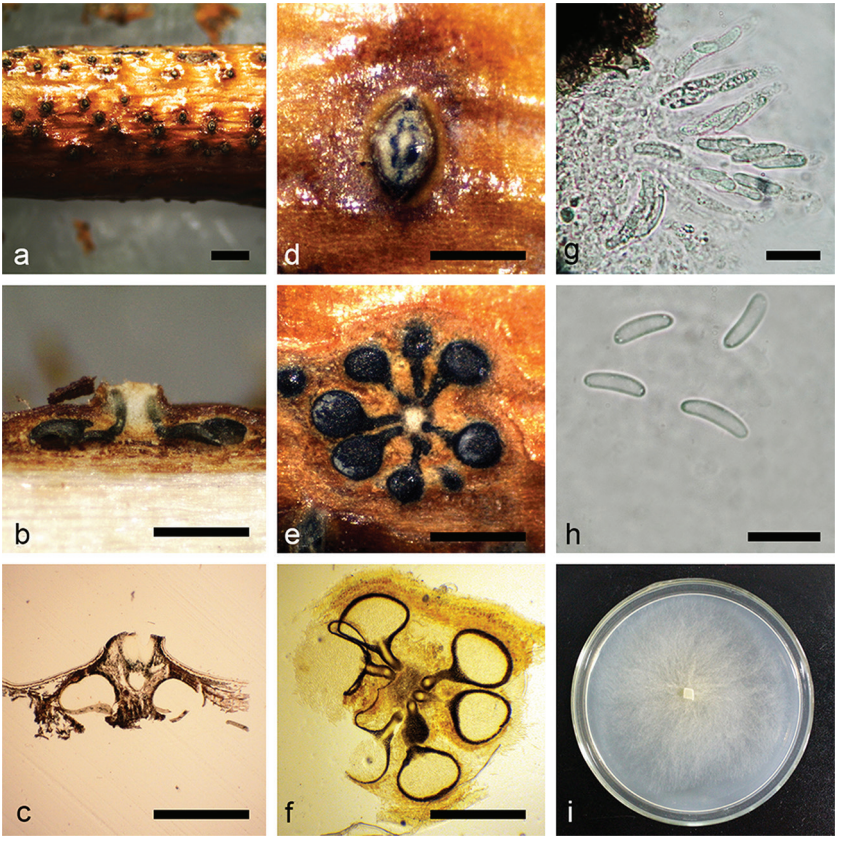
Ascostromata immersed in the bark, erumpent, circular to ovoid, 0.72–1.15 mm (x = 1.03 mm, n = 20) diameter, with a well defines black zone of demarcation, which was known as the conceptacle. Disc was usually obscured by tightly ostiolar necks, when apparent pale brown to beige, nearly flat, circular to ovoid, 0.19–0.27 mm (x = 0.24 mm, n = 20) in diameter, with 3–8 ostioles arranged circinately in a disc, brown to black. 3–8 perithecia arranged circinately in beige entostromata, flask-shaped to spherical, 0.23–0.31 mm (x = 0.28 mm, n = 20) in diameter. Asci free, clavate to elongate obovoid, 33.6–42.1 × 6.0–7.1 μm (x = 40.4 × 6.9, n = 20), 8-spored. Ascospores, elongate-allantoid, thin-walled, hyaline, aseptate, 10.7–14.8 × 2.4–2.9 μm (x = 12.9 × 2.6 μm, n = 50). Cultural characteristics were white originally, became dark brown and black after 14 d. The colony was flat, felt-like, and the texture was uniform.

Materials examined: **China**, **Gansu Province**, Gannan, Zhuoni, 34°35′16.64″N 103°09′39.53″E, elev. 2758 m asl, on stems of *Salix cupularis*, 8 August 2012, Y.M. Liang & X.L. Fan (BJFC-S730; culture BJFC-CGLs32; GenBank KC787364); Gannan, Zhuoni, 34°29′23.60″N 103°06′40.18″E, elev. 2540 m asl, on stems of *Salix cupularis*, 8 August 2012, Y.M. Liang & X.L. Fan (BJFC-S736; culture BJFC-VGLs33; GenBank KC787363).

Valsa populina (Pers.) Fuckel, Jahrb. Nassauischen Vereins Naturk. 25–26: 314, 1871.
Plate 5

Ascostromata immersed in the bark, erumpent, circular to ovoid, 0.90–1.27 mm (x = 1.07 mm, n = 20) diameter. Disc was usually obscured by tightly ostiolar necks, when apparent pale brown to beige, nearly flat, circular to ovoid, 0.19–0.24 mm (x = 0.22 mm, n = 20) in diameter, with 6–8 ostioles arranged circinately in a disc, brown to black. 6–8 perithecia arranged circinately in beige entostromata, flask-shaped to spherical, 0.25–0.26 mm in diameter. Asci free, clavate to elongate obovoid, 55.0–68.3 × 8.2–12.7 μm (x = 62.5 × 10.5 μm, n = 20), 4-spored. Ascospores, elongate-allantoid, thin-walled, hyaline, aseptate, 18.7–24.1 × 4.3–5.8 μm (x = 20.4 × 5.1 μm, n = 50). Cultural characteristics were white. The colony was flat, felt-like, and the texture was uniform. Conidiomata formed on PDA uniformly.

Material examined: **China**, **Gansu Province**, Gannan, Lintan, Yeliguan, 34°57′36.48″N 103°39′59.70″E, elev. 2181 m asl, on stems of *Salix matsudana* f. *tortuosa*, 5 August 2012, Y.M. Liang & X.L. Fan (BJFC-S716; culture BJFC-VGLs7; GenBank KC787366); Gannan, Pucigou, 34°04′51.38″N 103°23′34.33″E, elev. 3041 m asl, on stems of *Salix cupularis*, 9 August 2012, Y.M. Liang & X.L. Fan (BJFC-S725; culture BJFC-VGLs24; GenBank KC787367).



Pl. 5 *Valsa populina* (BJFC-VGLs7). **a** Ascomata on twig. **b, c** Longitudinal sections through ascomata. **d** Disc of ascomata on twig. **e, f** Transverse sections through ascomata **g** Asci and immature ascospores. **h** Ascospores. **i** Colonies on PDA at 3 d. Scale bars: a = 1 mm; b–f = 0.5 mm; g–h = 20 μm.

Discussion

Previously nine species of *Cytospora* and *Valsa* had been recorded on *Salix* in China. However, the identification of some of these species is questionable: the records of *C. capreae*, *C. salicis*, *C. fertilis*, *C. translucens*, *C. fugax*, and *C. germanica* share similar morphological characteristics but lack detailed descriptions and molecular analyses, and we agree that they should be unified under *C. fugax* (the name with priority) as suggested by Tai (1979). Consequently, based on earlier records only four species of *Cytospora* and *Valsa* from *Salix* can be accepted in northern China: *C. chrysosperma*, *C. fugax*, *C. ambiens*, and *V. populina*. In this study, we confirm the earlier reports of

C. chrysosperma and *V. populina* from *Salix* in China and add *C. nivea* as a new host record and *C. rostrata* as a new species.

Cytospora rostrata has a single locule without invaginations. *Cytospora pruinosa* (Fr.) Sacc., *C. eucalypticola* Van der Westh, *C. variostromatica* G.C. Adams & M.J. Wingf. also have single locules but differ from *C. rostrata* by having an ostiolar disc, rather than a thorn-like beak. All multi-locular species of *Cytospora* are further differentiated from *C. rostrata* by their lack of a thorn-like beak.

Phylogenetic analyses also support the proposal of *C. rostrata* as a new species. *Cytospora rostrata* has its closest genetic relationship with *C. chrysosperma*, which is the most widespread species of *Cytospora*.

Acknowledgments

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